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New Insights into the Regulation of Plant Succinate Dehydrogenase

ON THE ROLE OF THE PROTONMOTIVE FORCE*

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Charles Affourtit‡§, Klaas Krab¶, Graeme R. Leach§, David G. Whitehouse,
and Anthony L. Moore

From the Department of Biochemistry, School of Biological Sciences, University of Sussex, Falmer,
Brighton BN1 9QG, United Kingdom and the ¶Department of Molecular Cell Physiology, BioCentrum Amsterdam,
Faculty of Biology, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

Regulation of succinate dehydrogenase was investigated using tightly coupled potato tuber mitochondria in a novel fashion by simultaneously measuring the oxygen uptake rate and the ubiquinone (Q) reduction level. We found that the activation level of the enzyme is unambiguously reflected by the kinetic dependence of the succinate oxidation rate upon the Q-redox poise. Kinetic results indicated that succinate dehydrogenase is activated by both ATP ($K_{1/2} \sim 3 \mu\text{M}$) and ADP. The carboxyatractyloside insensitivity of these stimulatory effects indicated that they occur at the cytoplasmic side of the mitochondrial inner membrane. Importantly, our novel approach revealed that the enzyme is also activated by oligomycin ($K_{1/2} \sim 16 \text{ nM}$). Time-resolved kinetic measurements of succinate dehydrogenase activation by succinate furthermore revealed that the activity of the enzyme is negatively affected by potassium. The succinate-induced activation ($\pm K^+$) is prevented by the presence of an uncoupler. Together these results demonstrate that *in vitro* activity of succinate dehydrogenase is modulated by the protonmotive force. We speculate that the widely recognized activation of the enzyme by adenine nucleotides in plants is mediated in this manner. A mechanism that could account for such regulation is suggested and ramifications for its *in vivo* relevance are discussed.

Succinate dehydrogenase is a membrane-bound component of the respiratory chain of aerobic organisms (1). It couples the reduction of ubiquinone (Q)¹ to the oxidation of succinate and is, as such, a Krebs cycle as well as a respiratory chain enzyme. This duality suggests a potentially important role in the control of energy metabolism, a reason why the enzyme's regulation has been the subject of previous extensive investigations in both mammalian (2, 3) and plant (4, 5) systems.

The isolation of succinate dehydrogenase from mammalian sources yields a predominantly deactivated enzyme due to the

presence of tightly bound oxaloacetate (6, 7). The isolated enzyme, however, can be activated by substrates and substrate analogues (8) as well as by many anions, acid pH (9, 10), reducing treatments, (11) and QH_2 (2, 12). Additionally, succinate dehydrogenase is activated by ATP (3) via a mechanism that, even to date, is poorly understood. In the presence of ADP or an uncoupler, the mammalian enzyme is rapidly deactivated (3). Gutman *et al.* (3) attributed physiological relevance to these observations reasoning that deactivation of succinate dehydrogenase would benefit the cell when the ATP:ADP ratio is low, because it would allow faster NADH oxidation and hence more efficient ATP synthesis. The subsequently formed ATP would then activate the enzyme to allow turnover of the Krebs cycle to proceed in an unhindered fashion (3).

In plants, succinate dehydrogenase is activated by substrates, QH_2 , ATP, anions, and acid pH in a manner similar to that observed in mammalian systems (4, 5). The effect of ADP on the plant enzyme, however, has not been established conclusively. Oestreicher *et al.* (5) reported that in mung bean and cauliflower mitochondria, succinate dehydrogenase is activated rather than deactivated by ADP, and therefore they concluded that the physiological significance of ATP-induced activation in plants could not be the same as that proposed for mammalian systems. In *Arum maculatum* mitochondria, on the other hand, succinate dehydrogenase, similar to the mammalian enzyme, appears to be deactivated by ADP (13).

The incomplete understanding of the activation of succinate dehydrogenase by adenine nucleotides, particularly in plants, might be partly due to the techniques employed to measure this regulatory phenomenon. Traditional assays of the activation state of succinate dehydrogenase involve artificial electron acceptors (4, 5) or are based on oxygen uptake measurements (14–16). These methods both have limitations; the former cannot be readily used in well coupled mitochondria, whereas the latter does not yield data that reflect specific succinate dehydrogenase activity. Evidently, data that are obtained in uncoupled systems using non-endogenous substrates are not easily interpreted in terms of their potential physiological relevance. By measuring the oxygen uptake rate and, simultaneously, the reduction level of the Q-pool (*cf.* Ref. 17), information can be obtained as to the kinetic behavior of mitochondrial respiratory enzymes (including succinate dehydrogenase) toward their natural substrates within a tightly coupled environment. Such modular kinetic measurements have previously yielded valuable data concerning the kinetic interplay between Q-reducing and QH_2 -oxidizing enzymes both in isolated plant and yeast mitochondria (18–20). Mathematical modeling of these kinetic data is proving an increasingly powerful tool in understanding the *in vivo* role of mitochondrial electron transfer (18–23).

In this paper we have followed a modular kinetic approach to

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‡ To whom correspondence should be addressed. Tel.: 44-1273 872696; Fax: 44-1273 678433; E-mail: C.Affourtit@sussex.ac.uk.

¹ The abbreviations used are: Q, ubiquinone; Ap5A, P^1P^5 -di(adenosine-5')pentaphosphate; CAT, carboxyatractyloside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\psi$, mitochondrial inner membrane potential; MOPS, 3-(*N*-morpholino)propanesulfonic acid; QH_2 , reduced ubiquinone; SDH, combined ubiquinone-reducing activity of succinate dehydrogenase and the dicarboxylate carrier.

determine unequivocally the various activation states of succinate dehydrogenase in tightly coupled potato tuber mitochondria. It is confirmed that the enzyme is activated by both ATP and ADP, which, interestingly, appears to occur at the cytoplasmic side of the inner membrane. Importantly, our novel approach reveals that the activation state of succinate dehydrogenase is affected by oligomycin as well as by potassium. These observations suggest that *in vitro* activity of the plant succinate dehydrogenase can be modulated by the protonmotive force.

EXPERIMENTAL PROCEDURES

Fresh potato tubers were purchased in a local supermarket and stored overnight at 4 °C. Mitochondria were isolated and purified according to a previously described protocol (24) either in the presence or the absence of added K^+ . The isolation and purification media used in the respective procedures were buffered at pH 7.4 with either MOPS/KOH or MOPS/NaOH. Rat liver mitochondria were prepared from male Wistar rats in the presence of K^+ essentially as described elsewhere (25). Protein content was estimated using the bicinchoninic acid method with bovine serum albumin as a standard (26).

Respiratory activity and the reduction level of the Q-pool were simultaneously measured voltametrically in a specially constructed chamber (University of Sussex) housing a Rank oxygen electrode and glassy carbon and platinum electrodes connected to an Ag/AgCl reference electrode similar to that described by Moore *et al.* (17). In experiments with mitochondria isolated in the absence of K^+ , the reference electrode was connected via a NaCl, rather than a KCl, agar bridge. Mitochondria were incubated in 2.2-ml reaction medium (medium A) that contained mannitol (0.3 M), $MgCl_2$ (1 mM), K_2HPO_4 (5 mM), KCl (10 mM), MOPS (20 mM, pH adjusted to 7.2 with KOH), and ubiquinone-1 (1 μM). K^+ salts were replaced by Na^+ salts (medium B) in experiments with mitochondria isolated in the absence of K^+ . Other chemicals were added as indicated in the figure legends. Data were recorded digitally using a PowerLab/4SP system (ADInstruments Pty Ltd., UK) connected to iMac running Chart version 3.6s software (ADInstruments Pty Ltd.).

RESULTS

Similar to their mammalian counterparts, fresh potato tuber mitochondria contain only the cytochrome pathway through which reducing equivalents are transferred from QH_2 to oxygen. Upon oxidation of succinate, the respiratory chain in isolated potato mitochondria can therefore be considered to consist of two kinetic units that “communicate” through a single intermediate, the Q-pool. This pool is reduced by the combined action of succinate dehydrogenase and the dicarboxylate carrier (to allow succinate entry into the mitochondrial matrix) and subsequently oxidized by the cytochrome pathway (*cf.* Ref. 23). Activation of the Q-reducing side of the respiratory system (from this point simply referred to as SDH) will result in an increased rate of electron transfer and, concomitantly, in an increased reduction level of the Q-pool. On the other hand, stimulation of the QH_2 -oxidizing side will also cause an increased respiratory activity, but this will be accompanied by a decreased rather than an increased Q-reduction level. Simultaneous steady state measurement of the O_2 -uptake rate and the Q-redox poise will therefore readily reveal which side of the respiratory system is activated. This is clearly illustrated by the data shown in Fig. 1.

The addition of succinate to a reaction mixture containing potato tuber mitochondria results in a 20–35% reduction of the Q-pool and in an oxygen uptake rate of ~30 nmol of O_2 /min/mg (Fig. 1). The subsequent addition of ATP causes a slight increase in the respiratory rate and, significantly, a striking further reduction of the Q-pool (to ~80%). The addition of ADP results in a substantially increased rate (typical respiratory control ratio ~ 4) and in an oxidation of the Q-pool to ~60% (Fig. 1A), demonstrating that electron transfer is tightly coupled to phosphorylation. The considerable ATP-dependent rise in the Q-redox poise, together with the small but detectable

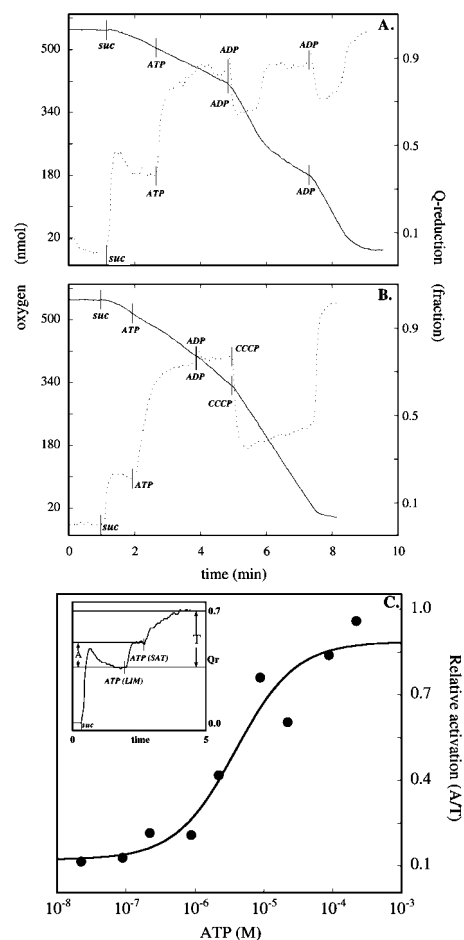


FIG. 1. Activation of SDH by ATP. Typical oxygen consumption (solid line) and Q-reduction (dotted line) traces are shown illustrating SDH activation by ATP in the absence (A) and the presence (B) of 5 μM CAT. Succinate (suc, 9 mM), ATP (0.2 mM), ADP (0.1 mM), and CCCP (1 μM) were added as indicated. Panel C, quantification of the ATP-induced activation (–CAT) in a manner illustrated by the Q-reduction trace shown in the inset. Succinate (suc, 9 mM) and ATP, either at a limiting (0.2 μM , LIM) or a saturating (0.2 mM, SAT) concentration, were added as indicated (see text for further details). Titration data were modeled with an expression of the form $y = (a \cdot x + b) / (c \cdot x + 1)$ where y represents the relative activation and x is the ATP concentration. Q-reduction (Q_r) is expressed as the fraction QH_2 in the pool. All assays were performed in medium A that contained ~ 0.6 mg of mitochondrial protein (from potato tuber).

increase in respiratory rate, confirms that SDH is activated by ATP. This activation is insensitive to CAT (Fig. 1B), an inhibitor of the mitochondrial adenine nucleotide translocator (27), which suggests that ATP exerts its stimulatory effect at the cytoplasmic side of the inner membrane. The addition of ADP in the presence of CAT does not significantly affect the respiratory rate or the Q-redox poise (Fig. 1B), confirming that CAT prevents ADP entry into the matrix. As anticipated, CCCP-induced uncoupling also causes an increase in the respiratory rate (~5-fold) as well as a substantial oxidation of the Q-pool (to ~35%; Fig. 1B).

ATP-induced activation of SDH has, to our knowledge, not been quantified to date. The relatively large and fast change observed in the Q-redox poise (Fig. 1, A and B), however, readily enables quantification (Fig. 1C). The level of activation can be defined as the difference between the Q-redox poise that is attained in the presence of succinate + ATP and the Q-reduction level that is reached in the presence of succinate alone (Fig. 1C, inset). A measure of relative activation is obtained by expressing the activation level observed at a limiting ATP concentration (A) as a fraction of that seen at a saturating

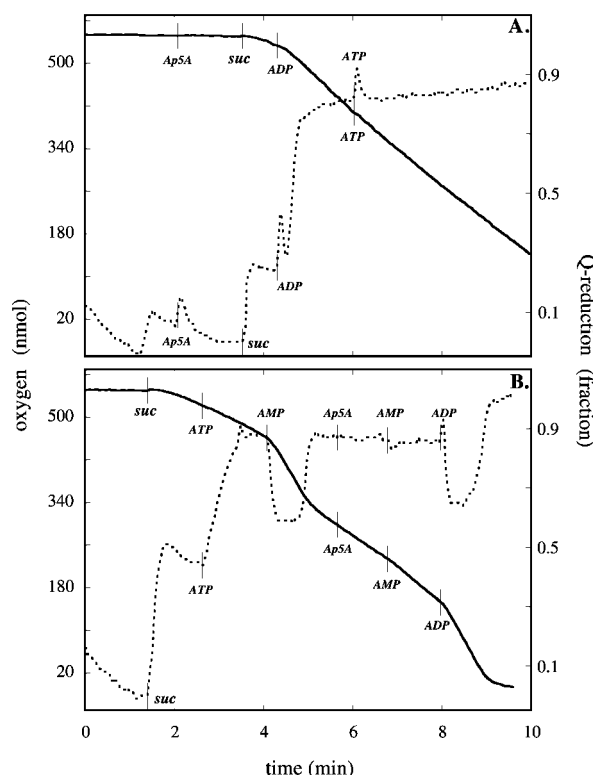


FIG. 2. **Activation of SDH by ADP.** Typical oxygen consumption (solid line) and Q-reduction (dotted line) traces are presented showing oxidation of succinate in the presence (A) or the absence (B) of 5 μ M CAT. As indicated, succinate (suc, 9 mM), ADP (0.2 mM), ATP (0.2 mM), AMP (0.1 mM), and Ap5A (9 μ M) were added to medium A that contained \sim 0.6 mg of mitochondrial protein (from potato tuber). Q-reduction is expressed as the fraction QH_2 in the pool. See text for further details.

concentration (T). The titration data presented in Fig. 1C show that SDH is activated half-maximally by \sim 3 μ M ATP.

It has been reported previously (5) that SDH in mung bean and cauliflower mitochondria is not only activated by ATP but also by ADP. The addition of ADP to fresh potato mitochondria that were pre-incubated in the presence of CAT and Ap5A (inhibitor of adenylate kinase (28)) caused an immediate and significant reduction of the Q-pool (from \sim 20 to \sim 80%) as well as an increase in the respiratory rate (Fig. 2A). This indicates that SDH is indeed activated by ADP. That this activation is affected neither by CAT nor by Ap5A shows that the effect is not due to indirect ATP formation through phosphorylation of ADP within the mitochondrial matrix or through adenylate kinase activity within the intermembrane space, respectively. These observations demonstrate that SDH activity is stimulated by ADP *per se*, which, similar to the activation by ATP, occurs at the cytoplasmic side of the inner membrane. Such an activation is in contrast with the observation that SDH is deactivated by ADP in *Arum* mitochondria (13). It should be noted, however, that the deactivation in *Arum* was observed in the absence of CAT. Addition of ADP under such conditions causes an oxidation of the Q-pool (*cf.* Fig. 1A) and most likely a decrease of the membrane potential ($\Delta\psi$) because of proton flux into the matrix through the ATP synthase (*cf.* Ref. 29). Therefore it cannot be excluded that the observed deactivation of SDH by ADP in *Arum* mitochondria is accounted for by these effects (*cf.* "Discussion"). The Q-redox poise attained in potato mitochondria upon the addition of ADP in the presence of CAT is not significantly affected by the subsequent addition of ATP (Fig. 2A). This suggests that both nucleotides activate SDH in a similar fashion. The respiratory trace shown in Fig. 2B con-

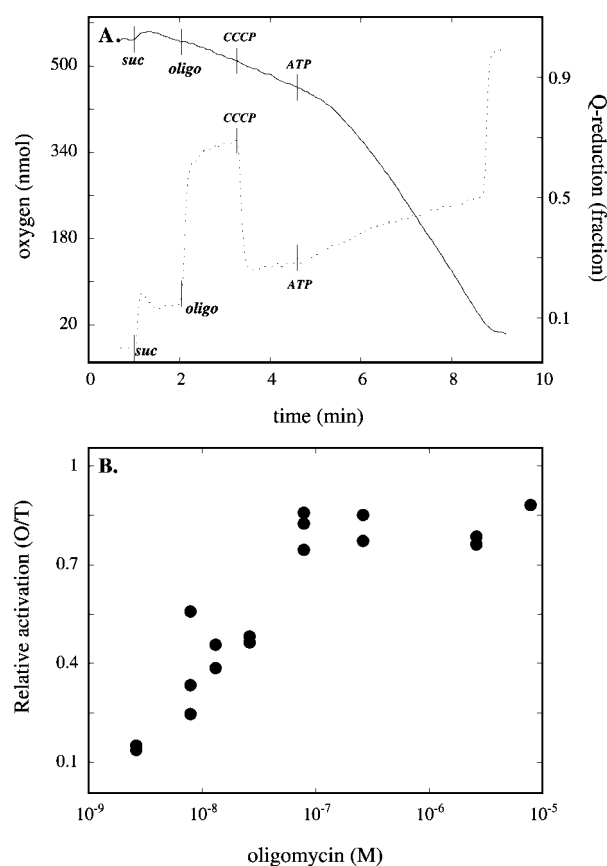


FIG. 3. **Activation of SDH by oligomycin.** Panel A, typical oxygen consumption (solid line) and Q-reduction (dotted line) trace to illustrate SDH activation by oligomycin. Succinate (suc, 9 mM), oligomycin (oligo, 3 μ M), CCCP (1 μ M), and ATP (0.2 mM) were added as indicated. Q-reduction is expressed as the fraction QH_2 in the pool. Panel B, quantification of the activation by oligomycin performed in a similar fashion as described for ATP (see Fig. 1C). The activation level attained with a limiting amount of oligomycin (O) is expressed as a fraction of that reached with a saturating amount of ATP (T) to obtain a measure of relative activation. All assays were performed in medium A that contained \sim 0.9 mg of mitochondrial protein (from potato tuber).

firms that Ap5A indeed inhibits adenylate kinase activity, because its presence prevents an AMP-induced state 3 (*cf.* Ref. 30).

The effects of adenine nucleotides may implicate the mitochondrial ATP synthase, even though activation of SDH by these compounds occurs in the presence of CAT. To assess this possibility, ATP-induced activation was studied in the presence of oligomycin. It was found that oligomycin does not affect the activation by ATP (data not shown). Importantly, oligomycin alone appears to activate SDH. This is illustrated by the data presented in Fig. 3A, which show that addition of oligomycin to mitochondria oxidizing succinate results in a considerable and immediate reduction of the Q-pool (to \sim 70%) and in a small but significant increase in the respiratory activity (from \sim 30 to \sim 60 nmol of O_2 /min/mg). Similar effects are observed when oligomycin is substituted by *N,N'*-dicyclohexylcarbodiimide, another inhibitor of the ATP synthase (data not shown). The subsequent addition of CCCP results in a strong oxidation of the Q-redox poise (to \sim 25%) and does not affect the oxygen uptake rate significantly. This indicates that the oligomycin-induced activation of SDH is reversed by CCCP. When ATP is added under these conditions, reactivation of SDH occurs, which is reflected in a gradually increasing respiratory rate and Q-reduction level (Fig. 3A). This reactivation, however, is significantly slower than the ATP-induced activation observed in the absence of oligomycin and CCCP (*cf.* Figs. 3A and 1A). In

Fig. 3B the oligomycin-induced activation is presented quantitatively and expressed as a fraction of the activation that is achieved in the presence of a saturating amount of ATP. It can be inferred from Fig. 3B that activation of SDH by oligomycin is not to the same extent as that by ATP (maximal activation by oligomycin is $\sim 85\%$ of the stimulation observed at a saturating ATP concentration) and that half-maximal stimulation occurs at ~ 16 nM. It has previously been reported that oligomycin causes an increase in $\Delta\psi$ when added to potato mitochondria oxidizing succinate under state 2 conditions (30). It is therefore likely that SDH is not directly activated by oligomycin but indirectly by an increased $\Delta\psi$. This notion agrees well with the observed deactivating effect of CCCP (Fig. 3A).

It has recently become apparent that plants contain a mitochondrial ion channel that mediates unidirectional K^+ entry into the matrix (31). The activity of this K^+ channel effectively de-energizes the inner membrane and, importantly, is inhibited by both ATP and ADP (31). Thus, when mitochondria are incubated in the presence of KCl, succinate alone is incapable of generating a $\Delta\psi$. However, $\Delta\psi$ is readily established in the additional presence of ATP or indeed in the absence of KCl (31). It is therefore conceivable that activation of SDH by adenine nucleotides, similar to that by oligomycin, is mediated by $\Delta\psi$. To explore this possibility further, activation experiments were performed with mitochondria isolated either in the presence or the total absence of K^+ .

From Fig. 4A (trace 1) it can be seen that the addition of succinate to mitochondria isolated in K^+ -containing media results in an initial Q-reduction level of $\sim 10\%$. Over a period of ~ 6 min the Q-pool becomes gradually more reduced (to $\sim 40\%$), indicating a relatively slow succinate-induced activation of SDH. Under these conditions, however, a steady state Q-redox poise (*i.e.* SDH activation level) is not reached before oxygen is exhausted. On the other hand, when succinate is added to mitochondria isolated in the absence of K^+ , a comparatively rapid increase in Q-reduction is observed (Fig. 4A, trace 2). Under these conditions, a steady state Q-redox poise ($\sim 80\%$) is achieved prior to anaerobiosis. Activation of SDH by succinate is almost completely prevented by CCCP, as evidenced by the low and steady Q-redox poise that is attained in samples either containing or lacking K^+ (Fig. 4A, traces 3 and 4, respectively). In both samples, the fastest and most substantial activation is observed in the presence of ATP, which is reflected by a Q-redox poise of $\sim 95\%$ reached within 1 min following the addition of succinate (Fig. 4A, traces 5 and 6).

In Fig. 4B data are presented from a typical experiment using mitochondria isolated in the absence of K^+ . It is clear that activation by succinate (Fig. 4B, trace 1) is significantly slower when KCl is added to the reaction mixture (Fig. 4B, trace 4). The resultant activation kinetics are indeed very similar to those observed in mitochondria isolated in the presence of KCl (*cf.* Fig. 4A, trace 1). Activation by succinate is also slow in the presence of NaCl (Fig. 4B, trace 3), albeit to a lesser extent than in the presence of KCl. Cholinechloride does not significantly affect the activation (Fig. 4B, trace 2) confirming that the observed effects are cation- rather than chloride-induced.

In rat liver mitochondria, the Q-pool is rapidly reduced to almost 100% upon the addition of succinate (Fig. 4C, trace 1; note that K^+ was present during isolation but absent in the assay). In this system, SDH is therefore fully activated by succinate alone, which is also apparent from the observation that ATP has no significant additional effect (Fig. 4C, trace 2). The activation by succinate (\pm ATP) appears to be even somewhat faster than the ATP-induced activation of SDH observed in potato tuber mitochondria, isolated \pm KCl (Fig. 4C, compare

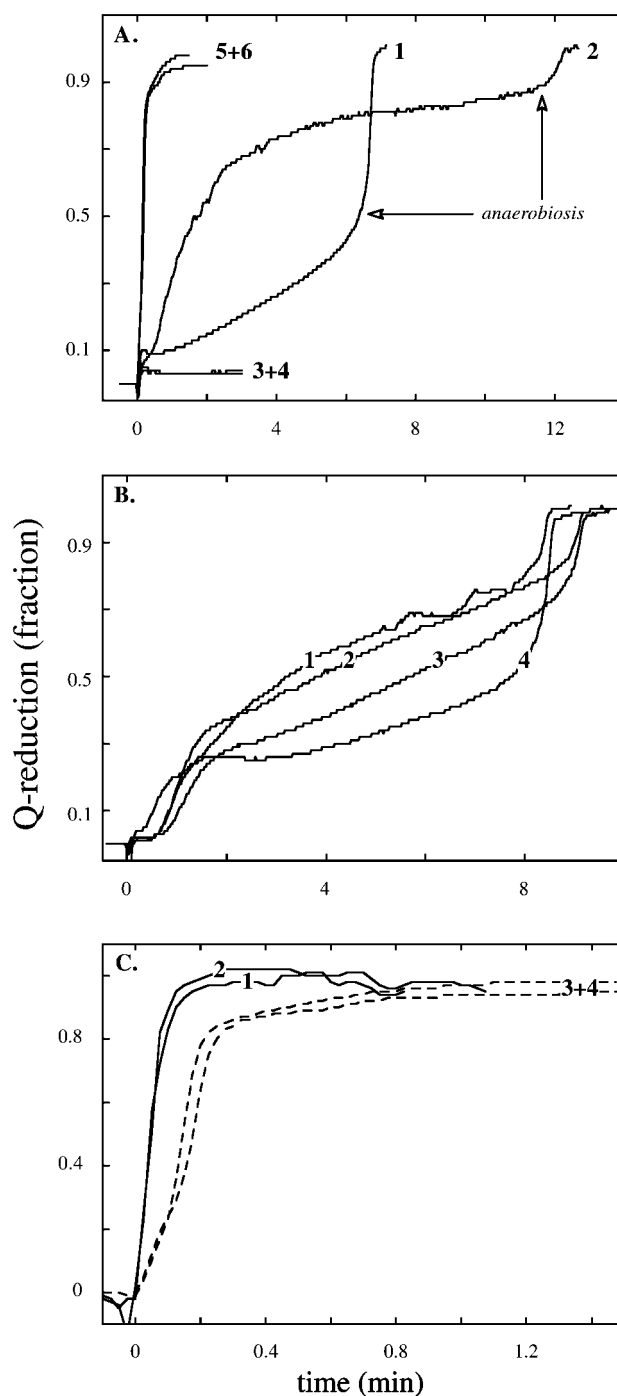


FIG. 4. Time-resolved activation of SDH. Typical Q-reduction traces to illustrate activation of SDH by succinate (9 mM) that was added at $t = 0$ in all assays. Q-reduction is expressed as the fraction QH_2 in the pool. Panel A, experiments 1, 3, and 5 were performed in medium A with potato mitochondria (~ 2.2 mg) isolated in the presence of K^+ , whereas experiments 2, 4, and 6 were carried out in medium B with potato mitochondria (~ 1.2 mg) isolated in the absence of K^+ ; CCCP (1 μ M) and ATP (0.2 mM) were present in assays 3+4 and 5+6, respectively. Panel B, all experiments were performed in medium B with potato mitochondria (~ 1 mg) isolated in the absence of K^+ ; cholinechloride, NaCl, and KCl (all at 0.18 M) were present in traces 2, 3, and 4, respectively. Panel C, experiments 1 and 2 were performed in medium A with rat liver mitochondria (~ 3.4 mg) isolated in the presence of K^+ ; ATP (0.2 mM) was present in assay 2; traces 3+4 are also presented on a different time scale in panel A (traces 5+6).

traces 1 and 2 with traces 3 and 4).

It is evident from the above results that Q-reduction measurements provide important information on SDH activation,

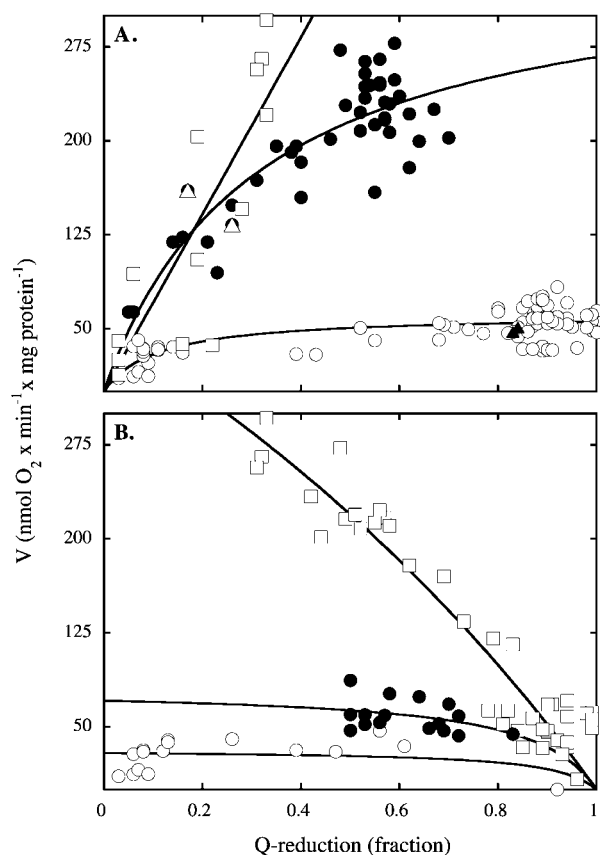


FIG. 5. Kinetic dependence of cytochrome pathway (A) and SDH (B) activity upon the Q-redox poise. Panel A, cytochrome pathway kinetics were determined by titrating succinate oxidation (9 mM) with malonate (0–4.5 mM), under state 4 (○), state 3 (●, 0.9 mM ADP), and uncoupled (□, 1 μ M CCCP) conditions. Also shown are steady states reached upon the oxidation of NADH (1.8 mM) under state 4 (▲) or state 3 (△) conditions. Panel B, SDH kinetics were determined by titrating succinate oxidation (9 mM) with antimycin A (0–0.2 μ M) in the absence (○) and in the presence of oligomycin (●, 3 μ M) or ATP (□, 0.2 mM). The kinetic data are an accumulation of more than 200 separate respiratory traces performed with 8 independent mitochondrial preparations (from potato tuber). Q-reduction is expressed as the fraction QH_2 in the pool, and the results were modeled according to Ref. 18.

particularly in terms of time-resolved kinetics. However, the Q-kinetic method is most powerful when Q-reduction levels are related to the corresponding oxygen uptake rates. Titration of the respiratory activity with an appropriate inhibitor thus reveals the kinetic dependence of both Q-reducing and QH_2 -oxidizing enzymes upon the reduction level of the Q-pool (18, 19, 21, 23).

In Fig. 5A the steady states reached upon oxidation of succinate in the presence of various malonate concentrations are shown, reflecting the kinetic behavior of the cytochrome pathway. It is clear that the rate of electron transfer through this pathway under state 4 conditions increases only slightly with the Q-redox poise. The presence of ADP alters these kinetics such that respiratory activity is substantially increased at any Q-reduction level. A further slight stimulation of the oxygen uptake rate, at Q-reduction levels greater than ~20% only, is observed when respiration is uncoupled by CCCP. Also shown in Fig. 5A are steady states that were attained upon oxidation of NADH both in the presence and the absence of ADP. It is clear that the NADH data are readily described by the appropriate kinetic curves fitted through the succinate data. This confirms that activity of the cytochrome pathway is kinetically independent of Q-reducing enzymes.

The kinetic behavior of SDH with respect to the Q-reduction

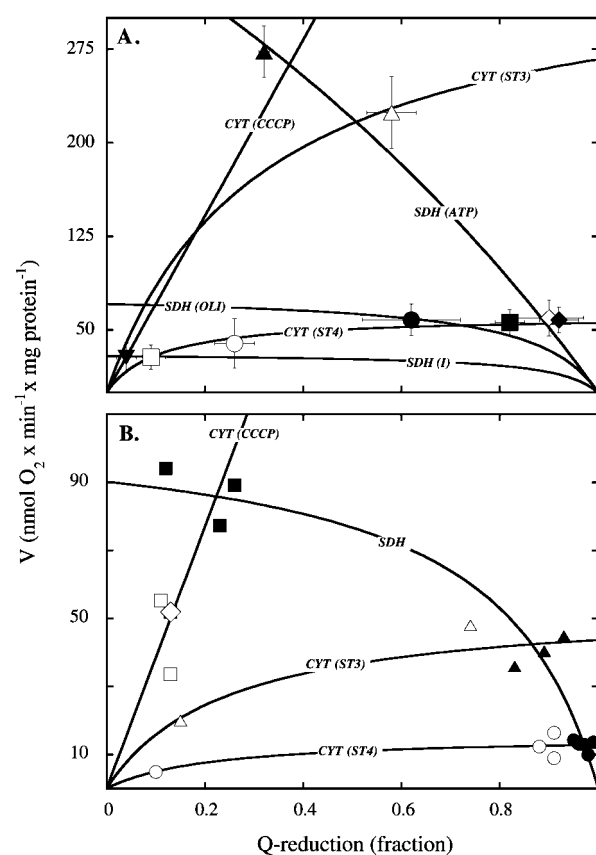


FIG. 6. Q-pool kinetics in mitochondria from potato tuber (A) and rat liver (B). Q-reduction is expressed as the fraction QH_2 in the pool. Panel A, steady states are shown that were reached (in mitochondria isolated $\pm \text{K}^+$ as indicated) upon oxidation of 9 mM succinate alone ($\pm \text{K}^+$ (□) or $-\text{K}^+$ (■)) or in the additional presence of either CCCP ($\pm \text{K}^+$, ▼, 1 μ M), oligomycin ($\pm \text{K}^+$, ●, 3 μ M), oligomycin + CCCP ($\pm \text{K}^+$, ○, 3 + 1 μ M), ATP ($\pm \text{K}^+$, state 2 (◇), or state 4 (◆), 0.2 mM), ATP + ADP ($\pm \text{K}^+$, △, 0.2 + 0.2 mM), or ATP + CCCP ($\pm \text{K}^+$, ▲, 0.2 mM + 1 μ M). The data are averages of 4–20 respiratory traces obtained from 8 independent mitochondrial preparations from potato tuber. The modeled curves are identical to those shown in Fig. 5 and represent cytochrome pathway kinetics under state 4 (CYT (ST4)), state 3 (CYT (ST3)), and uncoupled (CYT (CCCP)) conditions as well as SDH kinetics in an inactive (SDH (I)) and an oligomycin- or ATP-activated state (SDH (OLI) and SDH (ATP), respectively). Panel B, steady states achieved in a typical experiment with rat liver mitochondria oxidizing succinate (9 mM) under state 2 (●, ± 0.2 mM ATP), state 3 (▲, 0.9 mM ADP), or uncoupled (■, 1 μ M CCCP) conditions. The open symbols represent data obtained in the additional presence of malonate (0–4.3 mM) under state 2 (○), state 3 (△), and uncoupled (□) conditions. The steady state labeled as a diamond (◇) was attained upon addition of succinate (9 mM) to mitochondria pre-incubated with CCCP (1 μ M). Results were modeled according to Ref. 18, and the abbreviations used are the same as for panel A.

level was determined by titrating the succinate-dependent respiratory activity with antimycin A. From Fig. 5B it is evident that these kinetics are clearly affected by ATP such that SDH activity is stimulated at any Q-redox poise. A similar, although less substantial, effect on SDH kinetics is exerted by oligomycin. Both observations clearly demonstrate that Q-kinetic measurements unequivocally establish the different activation levels of SDH under tightly coupled (*cf.* Fig. 5A) conditions.

The curves that describe the kinetics of SDH and the cytochrome pathway as well as the steady states reached upon oxidation of succinate in the absence of malonate and antimycin A are displayed together in Fig. 6A. Several aspects concerning SDH activation are further clarified by this Q-kinetic representation. First, the steady state achieved in the presence of oligomycin and CCCP (Fig. 6A, ○) is closer to the kinetic

curve representing the inactive SDH (Fig. 6A, *SDH (I)*) than to the curve describing the oligomycin-activated enzyme (Fig. 6A, *SDH (OLI)*); this corroborates the earlier observation (*cf.* Fig. 3A) that activation by oligomycin is reversed by CCCP. Second, a notable difference can be observed between the steady states reached upon oxidation of succinate alone in mitochondria isolated either with K^+ (Fig. 6A, \square) or without K^+ (Fig. 6A, \blacksquare). The presence of K^+ results in a steady state (\square) that is perfectly modeled by the inactive SDH curve (Fig. 6A, *SDH (I)*), whereas the absence of K^+ causes the respiratory state (\blacksquare) to shift toward a position that is better described by the active SDH curve (Fig. 6A, *SDH (ATP)*). Note, however, that a (small) difference does exist between the succinate-induced steady state ($-K^+$; Fig. 6A, \blacksquare) and that attained in the presence of ATP ($\pm K^+$; Fig. 6A, \diamond). Finally, it is evident that CCCP effectively prevents the activation of SDH by succinate ($\pm K^+$; Fig. 6A, \blacktriangledown). Importantly, all these steady states are modeled accurately by the curves representing the kinetics of the cytochrome pathway under state 4 (Fig. 6A, *CYT (ST4)*) or uncoupled (Fig. 6A, *CYT(CCCP)*) conditions, confirming that it is not the activity of this pathway but indeed that of SDH that is modulated by oligomycin and K^+ .

For comparative reasons and because, to our knowledge, this information has not yet been reported, we also studied Q-pool kinetics in rat liver mitochondria (Fig. 6B). Although relatively few mammalian data were obtained, it is clear that this system is somewhat different from that in potato. Most notable are the differences in the specific (*i.e.* per milligram of mitochondrial protein) oxygen uptake rates, which, in rat liver, are approximately four (state 4), five (state 3) and three (+CCCP) times lower than in potato tuber. Oxygen uptake rates on a cellular basis, however, are substantially lower in potato tuber than in rat liver, because the latter yields roughly 50 times as many mitochondria per gram of tissue (data not shown). This would indicate, as anticipated, that rat liver tissue is metabolically more active than potato tuber.

Of particular interest is the finding that ADP affects the state 4 kinetics in rat liver in a similar manner to that seen in potato tuber but that the increase in respiratory rate, at any Q-redox poise, is considerably less substantial (Fig. 6B). This indicates that rat liver mitochondria contain inner membranes that are relative impermeable to protons even in the presence of ADP. This is confirmed by the observation that the presence of CCCP results in a further drastic increase in the oxygen uptake rate, again at any reduction level of the Q-pool (Fig. 6B).

The data shown in Fig. 6B furthermore illustrate that the rat liver SDH is fully activated by succinate alone, because the steady states attained in the presence and the absence of ATP are identical (Fig. 6B, \bullet). Interestingly, the steady state that is achieved by adding succinate after pre-incubation with CCCP (Fig. 6B, \diamond) is not at all satisfactorily modeled by the kinetic SDH curve, although it is adequately described by the curve reflecting uncoupled cytochrome pathway kinetics (Fig. 6B). The position of this steady state would suggest that pre-incubation with CCCP prevents a complete activation of SDH by succinate.

DISCUSSION

In this paper we have demonstrated how a modular Q-kinetic approach can improve our understanding of the regulation of mitochondrial respiration. It is evident that determination of the kinetic dependence of respiratory activity on the Q-reduction level unequivocally reveals the different activation levels attained by SDH (Figs. 5B and 6). It is also clear that continuous Q-reduction measurements provide valuable information on the time-resolved kinetics of SDH activation (Fig. 4). Importantly, activation of SDH was studied within a tightly

coupled environment (Fig. 5A) in which the enzyme reacts with its natural substrate (Q) and product (QH_2). Under such conditions, the Q-redox poise is significantly increased as a result of SDH stimulation, whereas the respiratory rate is hardly affected (*e.g.* Fig. 1). The sensitivity of the Q-reduction signal has enabled the quantification of ATP-induced SDH activation (Fig. 1C) and, more importantly, has revealed that the enzyme is activated by oligomycin (Fig. 3), a phenomenon that has remained unreported until now.

An interesting feature of SDH activation by ATP is its insensitivity to CAT, suggesting that activation occurs at the cytoplasmic side of the inner membrane (Fig. 1). This interpretation is not fully conclusive, however, because ATP may enter the matrix via a CAT-insensitive nucleotide carrier (32). SDH is also activated by ADP, again in a CAT-insensitive manner (Fig. 2). In the presence of CAT, ADP does not induce a state 3, which unambiguously confirms that it does not enter the matrix. Because ATP formation through adenylate kinase action has been excluded (Fig. 2), these data indicate that SDH is activated by ADP *per se* at the cytoplasmic side of the inner membrane. This strengthens the notion that activation by ATP also occurs in the intermembrane space, because it is intuitively unlikely that ADP and ATP activate SDH by different mechanisms.

The results presented in this paper disclose an important general aspect of (*in vitro*) mitochondrial SDH regulation, namely that the initial activation level of the enzyme is dependent on the source of the mitochondria as well as on the procedure employed to isolate them. SDH is only slowly activated by succinate in potato tuber mitochondria isolated in the presence of K^+ , whereas activation occurs faster and to a greater extent in organelles isolated in the absence of this cation (Fig. 4A). In both potato samples, activation by succinate is positively affected by ATP (Fig. 4A), whereas such activation is ATP-independent in rat liver mitochondria (Fig. 4C). These observations indicate that the initial activation level of SDH, upon mitochondrial isolation, is higher in rat liver than in potato and that the presence of K^+ during the preparation significantly lowers the activation level in potato tuber mitochondria.

Activation of SDH by succinate is prevented by CCCP, completely in potato (Fig. 4A) and to a large extent in rat liver (Fig. 6B) mitochondria. This strongly suggests that this activation is mediated by the protonmotive force. That SDH activity can be modulated in this fashion is corroborated by the observed stimulatory effect of oligomycin, which is reversed as well as prevented by CCCP (Figs. 3A and 6A). Earlier publications further support this novel regulatory mechanism. For example, extraction of succinate dehydrogenase from its native membrane results in a considerable decrease in the turnover number of the enzyme (33–35). Upon membrane reconstitution this number is restored to its original value, a result that has been interpreted as a positive modulation of succinate dehydrogenase activity by the membrane or one of its components (33–35). It is also apparent that activation by succinate occurs much more rapidly in tightly coupled mitochondria (from rat liver) than in membranous or soluble enzyme preparations (3). Moreover, the activation energy of the process by which NAD^+ -linked substrates (via generation of QH_2) activate succinate dehydrogenase is relatively low in intact mitochondria (3). It has been suggested that protein conformational changes, likely required for activation, are facilitated within a coupled environment (3, 8).

The data presented in this paper remain inconclusive as to whether activation of SDH by adenine nucleotides is also wholly mediated by the protonmotive force. However, several

lines of circumstantial evidence suggest that this could indeed be the case.

From our results it is clear that activation by ATP is slower in the presence than in the absence of CCCP (Figs. 3A and 1A), which suggests that activation occurs more readily within a coupled environment. This observation agrees well with the earlier findings that activation of succinate dehydrogenase by adenine nucleotides in cauliflower mitochondria is negatively affected by sonication or freeze-thawing treatments (5) and that the mammalian succinate dehydrogenase is not at all activated by ATP when studied in a solubilized form or in sub-mitochondrial particles (3). The fact, however, that SDH is activated in the presence of CCCP, albeit relatively slowly (Fig. 3A), could indicate an additional stimulatory effect of ATP that is not mediated via the protonmotive force.

As mentioned before under "Results," a discrepancy appears to exist between the SDH-activating effect of ADP in potato tuber mitochondria (Fig. 2) and the SDH-deactivating effect of this nucleotide in *A. maculatum* (13). The potato experiments were performed in the presence of CAT. Under such conditions $\Delta\psi$ is anticipated to remain relatively high upon the addition of ADP, thus allowing a ready activation of SDH. In the *Arum* study, on the other hand, CAT was absent, allowing ADP to enter the matrix where it is phosphorylated to ATP. Under those conditions the magnitude of $\Delta\psi$ is expected to drop (29). The finding that SDH activity in *Arum* mitochondria is not stimulated by ADP or ATP at a low $\Delta\psi$, but instead is deactivated, is in accordance with the relatively slow ATP-induced activation of SDH observed in potato mitochondria under uncoupled conditions (*cf.* Fig. 3A).

It has been reported that potato tuber mitochondria become relatively de-energized upon isolation, such that a membrane potential is not established prior to the addition of a respiratory substrate (see *e.g.* Ref. 30). Rat liver mitochondria, on the other hand, retain a comparably high level of matrix co-factors upon isolation and, as a consequence, remain in a relatively energized state. This is reflected by an initial (low) rate of rotenone-sensitive respiration which gradually vanishes within ~ 1 min from the start of the experiment (data not shown). The variable energy state of the respective preparations is also reflected by differences in proton permeability of the inner membrane. The addition of CCCP under state 4 conditions results in a 4.8- and 6.6-fold stimulation of respiratory activity in potato and rat liver mitochondria, respectively (Fig. 6, A and B). Interestingly, the different energization states correlate positively with the observed differential effect of ATP. In the relatively poorly energized potato tuber mitochondria, ATP is an absolute prerequisite to achieve maximum activation of SDH (Fig. 4A). In the more energized rat liver organelles, on the other hand, succinate alone is sufficient to accomplish this level of activation (Fig. 4C). This would suggest that ATP is not required for maximum SDH activation under coupled conditions and, in turn, that ATP exerts its stimulatory effect by increasing the protonmotive force.

As previously discussed, SDH activation by both ATP and ADP is likely to occur in the intermembrane space. It is difficult to envisage how these nucleotides would act directly on SDH at this side of the inner membrane. Several mechanisms, however, have been reported by which ATP increases $\Delta\psi$ (and hence the protonmotive force) when added to plant mitochondria oxidizing succinate under state 2 conditions. An increase in $\Delta\psi$ is, for example, induced by ATP through its inhibitory action on the mitochondrial plant uncoupling protein (36, 37). The inhibition of this uncoupling protein is unaffected by CAT and in that respect is similar to the stimulatory effect of ATP on SDH (Fig. 1B). However, it is unclear whether SDH activation

by ADP could be explained by this mechanism, because ADP has no reported effect(s) on the plant uncoupling protein.

Both ATP and ADP could potentially increase $\Delta\psi$ through their respective inhibitory actions on the mitochondrial K^+ channel (38) that recently has also been identified in plants (31). In the presence of KCl, plant mitochondria are effectively de-energized by the activity of this channel such that succinate alone is not able to generate a $\Delta\psi$ (31). In the additional presence of ATP, however, a $\Delta\psi$ is readily established in a manner that is insensitive to atractyloside (31). The plant K^+ channel is half-maximally inhibited by ATP at $\sim 290 \mu\text{M}$ (31), a concentration that is two orders of magnitude higher than that required to stimulate SDH (Fig. 1C). However, the ATP concentration needed to inhibit the mammalian K^+ channel half-maximally is only $\sim 2.3 \mu\text{M}$ (38), a value that is in striking accord with that calculated from Fig. 1C (*i.e.* $3 \mu\text{M}$). It should be noted that swelling experiments, performed in the mammalian system, are more suited to calculate rates of solute transport across the inner membrane than the $\Delta\psi$ -increment measurements made in the plant system (*cf.* Refs. 25 and 39). The mammalian K^+ channel is half-maximally inhibited by ADP at $\sim 0.5 \mu\text{M}$ (38). This relatively potent inhibition by ADP agrees well with our observation that SDH is activated to a higher extent by ADP (in the presence of CAT) than by an equimolar amount of ATP (data not shown). Although not conclusively shown, it is conceivable that ATP and ADP activate SDH indirectly by inhibiting the plant K^+ channel and thereby increasing $\Delta\psi$. This would be in line with the observation that the activation level of SDH is modulated by K^+ (Fig. 4, A and B).

As mentioned before, the Q-pool in potato mitochondria oxidizing succinate is reduced by the combined action of the dicarboxylate carrier and succinate dehydrogenase (*i.e.* SDH activity). From Q-kinetic *in vitro* measurements it is therefore not clear *a priori* whether a stimulatory effect on SDH is exerted at the level of succinate transport or at the level of succinate oxidation. *In vivo*, succinate is generated intra-mitochondrially, and hence an activating effect on transport would have little physiological relevance. It is conceivable that the observed regulation of SDH by $\Delta\psi$ occurs at the level of succinate entry, because this process is affected by the energy status of the inner membrane (40). It has been observed previously, however, that SDH activation by adenine nucleotides in cauliflower mitochondria is negatively affected by sonication or freeze-thawing treatments (5). In thawed or sonicated mitochondria, succinate should readily reach the active site of succinate dehydrogenase without the need of the dicarboxylate carrier. Such observations would therefore suggest that SDH is regulated at the level of succinate oxidation. It is arguable in general, however, whether regulation by $\Delta\psi$ has any *in vivo* importance at all, even if it would be exerted at the level of succinate oxidation. The reason for this is that it is highly unlikely that the severe degree of de-energization, brought about by addition of CCCP or indeed by the relative force required to isolate potato tuber mitochondria, would ever occur within the plant cell under physiological conditions.

In conclusion, we have shown that *in vitro* SDH activity can be regulated by the energy status of the mitochondrial inner membrane. The results suggest that the widely recognized activation of succinate dehydrogenase by ATP may also be the indirect result of such regulation. This implies that serious caution should be taken when physiological meaning is attributed to the regulation of succinate dehydrogenase by adenine nucleotides, both in plant and mammalian systems.

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